

Requirement for Chemotaxis in Pathogenicity of *Agrobacterium tumefaciens* on Roots of Soil-Grown Pea Plants†

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***Agrobacterium tumefaciens* Tn5 mutants deficient in chemotaxis to root exudates were used to study the significance of chemotaxis in crown gall pathogenesis. Mutants deficient in motility and in chemotaxis were fully virulent when inoculated by direct immersion in inoculum, followed by growth for 2 weeks in moist growth pouches. Ability of mutant bacteria to move through soil to infect roots was tested by planting wounded seedlings into air-dried soil or sand that had been infested with inoculum. Mutant bacteria were almost as virulent as the parent on plants grown in sand but were avirulent on soil-grown plants.**

Chemotaxis in bacteria involves the directed movement of motile cells in response to a gradient of attractant (1). The phenomenon in *Escherichia coli* and *Salmonella typhimurium* has been used extensively as a model system for investigating the molecular bases of how organisms detect and process sensory information (reviewed in references 19, 22, and 26). However, little is known about the role of this complex behavior in microbial ecology (23). Although the existence of chemotaxis in plant-associated bacteria is well documented (see, for example, references 5, 10, 14, and 24), studies on the biological significance of the phenomenon have been limited. The most complete studies have been carried out with *Rhizobium* species. The results indicate that nonchemotactic mutants of *Rhizobium* species can nodulate host roots, but their efficiency and competitiveness are reduced (2, 6, 9, 21). Conclusions about the role of chemotaxis in root colonization by other bacteria have been contradictory. For example, de Weger et al. (12) recently reported that motility of the plant growth-promoting pseudomonad *Pseudomonas fluorescens* is not required for colonization of potato seedlings but that colonization is greatly reduced in mutants without flagella. In contrast, two other research groups (20, 27) demonstrated that nonmotile mutants or strains of *P. fluorescens* colonized seedlings as effectively as did motile strains.

We are using *Agrobacterium tumefaciens* to test the role of chemotaxis in recognition between soil-borne pathogens and their hosts. *A. tumefaciens* is a gram-negative bacterium that infects plants through wounds in the root and root crown to cause a tumorigenic disease called crown gall (reviewed in references 7 and 29). Various *Agrobacterium* biotypes have been found in diverse environments, and the bacteria can invade cultivated soils and cause epidemics (8, 11). Despite the rapid progress that has been made in understanding the molecular biology of virulence in this pathogen (reviewed in references 7 and 29), little is known about how *A. tumefaciens* recognizes host tissue under natural conditions. We demonstrated that *A. tumefaciens* cells are attracted to root exudates of pea, and we selected Tn5 mutants that do not recognize the chemical signals from these complex substrates (18). In this study, the mutants were exploited to study the significance of the phenomenon in crown gall pathogenesis.

MATERIALS AND METHODS

Mutants. Tn5 mutants were generated from strain A348 as described previously (18). Excised root tips were used as a source of attractants from wounded tissue, referred to as wound exudates; suspensions of isolated root cap cells were used as a source of nonwound exudates. Cells of the root cap are the primary source of exudates that surround roots of healthy plants (4). Mutants that did not migrate toward exudates in a directional swarm assay were categorized according to phenotype. Motility mutants include two types: nonmotile mutants (C1669, C1530, C563, and C710), which failed to move at all from the center of the plate, and a slow-migrating mutant (C1786), which exhibited chemotaxis to root exudates but in a response apparent only after 48 to 72 h instead of the normal 24 h. Chemotaxis mutants exhibited several phenotypes. (i) A generally nonchemotactic mutant (C461) did not exhibit chemotaxis to any tested chemicals or exudates. (ii) Several mutants with normal (C503 and C531) or reduced (C294) motility did not recognize signals from wound or nonwound exudates but were attracted to some purified chemicals. (iii) A wound-specific mutant (C586) was attracted to wound but not nonwound exudates (18).

Growth of mutants. Growth rates were tested as follows. Standardized samples from log-phase cultures were added to 125 ml of liquid yeast extract-mannitol medium in 250-ml flasks. Bacterial concentration was tested at intervals by dilution plating onto solidified nutrient medium. To test the ability of mutants to multiply by using nutrients in root exudates, bacteria were added to a 100- μ l suspension of water containing approximately 5,000 isolated root cap cells, as described previously (15, 16). Bacterial concentration was tested by dilution plating after overnight incubation.

Virulence of mutants (in vitro). A growth pouch assay was used to measure virulence of mutants in vitro (17). Seedlings with root lengths of 10 to 20 cm were wounded at two sites: at the crown and within 1 to 2 mm of the root tip. Roots were immersed for 5 min in inoculum at different concentrations and then were planted in growth pouches. Infection was rated after 2 weeks by the percentage of inoculation sites that developed tumors (17).

Soil tumorigenesis assay. Assays were carried out in Cone-tainers (Ray Leach Nursery, Canby Oreg.) filled with 160 g of sterilized coarse sand or 100 g of sterile soil (sterility was confirmed by plating samples onto nutrient medium). The test soil is a silt loam (pH 7.5; 20% clay, 50% silt, 15% sand)

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from the Campbell Avenue Farm of the University of Arizona, Tucson. Two alternative inoculation procedures were used. In the first, direct inoculation, 10 ml of water containing bacteria adjusted to appropriate levels (based on number of colony-forming units per gram of soil or sand) was added directly to saturated sand or soil in containers. Wounded seedlings were planted into the infested soil within 20 min. In the second procedure, indirect inoculation, inoculum was incorporated into sand or soil by thorough mixing by hand on trays; the samples were then allowed to air dry in shallow layers (15 to 30 mm) for 48 h before the containers were filled. Wounded seedlings were then planted, and the samples were wetted up from the bottom by placing the containers (in racks) into water. For both treatments, inoculated plants were maintained in growth chambers at temperatures of 24°C (day) and 21°C (night), and water potential was kept constant at 10 mb (container tips were immersed to a depth of 1 cm). Infection was evaluated after 2 weeks. Bacterial viability was tested by plating soil samples after a 10-s sonication and was found to be stable over the test period, as demonstrated previously (16). Plants were removed from soil and washed thoroughly, and each inoculation site was compared visually with corresponding sites on control plants inoculated with parent strain A348, with Ti plasmidless strain A136, and with positive control Tn5 mutant 3057 or 1718. Transformation was confirmed by hybridization of DNA from selected plants with a probe from the conserved region of the T-DNA from strain C58 (13).

RESULTS

Growth and colony morphology of mutant bacteria. Growth rates of all but two mutants were indistinguishable from that of parent strain A348. Growth rates of the chemotaxis mutants C503 and C531 were slightly reduced in nutrient medium (data not shown). After 16 h in a suspension of root cap cells, these two mutants increased in numbers by only 10-fold. All other mutants and the parent strain increased in numbers by at least 100-fold, from approximately 2.0×10^6 up to 2.4×10^8 to 3.1×10^8 . On several types of culture medium, colony morphology of all but one of the mutants was identical to that of the parent strain. Colonies of mutant C294 were nonmucoid.

Virulence of mutant bacteria in vitro. As previously reported (17), the percentage of wound sites that developed tumors in response to inoculation with strain A348 was proportional to inoculum concentration (Table 1). All but three of the mutants exhibited wild-type virulence. One mutant, C294, was avirulent, and C531 and C503 produced fewer tumors at nearly all inoculum concentrations. Thus, reduced virulence in the growth pouch assay occurred only with the three mutants that exhibited phenotypic changes in growth or colony morphology in addition to chemotaxis defects.

Soil tumorigenesis assay. Preliminary assays indicated that A348 caused tumors on 50% of plants at a concentration of 10^6 bacteria per g of soil (data not shown), and this level was used in all subsequent assays. Virulence of five motility and five chemotaxis mutants was tested by direct inoculation of seedlings in soil. As in the growth pouch assay, most mutants induced tumors as effectively as A348, but C503 and C531 had reduced virulence, and C294 was avirulent (data not shown). Because it is possible that impaired virulence of these three mutants was due to something other than a direct effect on chemotaxis genes, they were not included in subsequent assays.

TABLE 1. Virulence of chemotaxis and motility mutants: growth pouch assay

Strain	% Tumorigenesis ^a at bacterial concn (log 10) of:					
	9	8	7	6	5	4
Positive controls						
Parent A348	96	92	80	47	8	6
Tn5 mutant						
3057	100	100	90	47	26	6
1718	87	93	77	30	11	3
Motility-deficient mutants						
Nonmotile						
C1669	87	83	70	40	10	6
C1530	97	97	77	66	43	16
C563	100	90	71	40	20	3
C710	93	86	58	13 ^b	10	0 ^b
Slow-migrating C1786	97	93	77	30	13	3
Chemotaxis-deficient mutants						
C294	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b	0 ^b
C461	99	97	77	53	10	10
C503	43 ^b	70 ^b	23 ^b	10 ^b	0 ^b	0 ^b
C531	50 ^b	37 ^b	27 ^b	30	0 ^b	0 ^b
C586	85	90	66	30	13	6

^a Fraction of inoculation sites that developed visible tumors. Values are means from at least 60 plants in three to five independent experiments.

^b Significantly different (0.05% confidence level) from the parent value at the designated inoculum level. Standard errors ranged from 5 to 15% of the mean.

Mutants that exhibited wild-type virulence in the growth pouch assay were selected for studies of behavior in soil and in sand (Table 2). In direct inoculation assays, the percentage of infection of seedlings by A348 was approximately 50% both in soil and in sand. Values for mutants on plants in sand were very similar to those of the parent, ranging from 46 to 50%. In soil, values for plants directly inoculated with mutant bacteria were slightly lower, 35 to 42%, but the values were not statistically distinct from those of the parent.

For indirect inoculation experiments in sand, tumorigenesis in response to parent bacteria was indistinguishable from results of direct inoculation, with 50% of plants forming tumors (Table 2). A slight reduction in tumorigenesis by mutant bacteria on plants in sand was statistically significant for only two of the mutant strains.

In indirect inoculation assays in soil, virulence of A348 was reduced, but nearly 20% of inoculated plants still formed tumors (Table 2). In contrast, all mutants were avirulent. Tumors failed to develop in response to mutant bacteria even when the inoculum level was increased 10-fold (data not shown).

DISCUSSION

In an in vitro assay that provided optimum exposure of bacteria to wound sites, virulence of all but 3 of 10 mutants was indistinguishable from that of the parent strain. The fact that seven different motility and chemotaxis mutants exhibited wild-type virulence indicates that these functions per se are not required for tumorigenesis on plants in growth pouches. The three mutants with reduced virulence had defects apart from loss of chemotaxis, including reduced growth rates and nonmucoid colony morphology. A reduced ability to multiply in wound sites would be expected to impair virulence. We have not ruled out the possibility that the insertion in C294 could overlap with a gene known to interfere with virulence. However, the wild-type virulence in

TABLE 2. Virulence of chemotaxis and motility mutants: direct or indirect inoculation in sand or in soil

Strain	% Tumorigenesis ^a			
	Direct inoculation ^b		Indirect inoculation ^c	
	Sand	Soil	Sand	Soil
Positive controls				
Parent A348	43 ± 10 (100)	46 ± 12 (100)	50 ± 5 (100)	18 ± 8 (100)
Tn5 mutant 1718	39 ± 8 (91)	41 ± 6 (95)	44 ± 8 (88)	15 ± 8 (83)
Motility-deficient mutants				
Nonmotile C1669	46 ± 7 (107)	42 ± 3 (98)	31 ± 9 ^d (62)	0
Slow-migrating C1786	48 ± 3 (111)	37 ± 8 (86)	36 ± 6 (72)	0
Chemotaxis-deficient mutants				
C461	50 ± 5 (104)	35 ± 6 (76)	38 ± 9 (76)	0
C586	48 ± 8 (111)	38 ± 10 (88)	33 ± 8 ^d (66)	0 ^d

^a Values represent means of at least 40 replicate treatments in each of two independent experiments. Each number in parentheses is the percentage of the value obtained with the parent in the designated treatment.

^b Wounded seedlings were planted in soil within 20 min of addition of 10 ml of inoculum to a well in the surface of the soil.

^c Wounded seedlings were planted in soil that had been infested with inoculum, blended thoroughly, and then air dried for 2 days before the experiment.

^d Significantly different from the parent value.

most mutants suggests that the impaired virulence in C503, C531, and C294 could well be secondary effects of mutations in genes that are not directly involved in chemotaxis. Direct inoculation of plants in sterile soil confirmed results obtained with plants in growth pouches: only C503, C531, and C294 exhibited virulence that differed significantly from that of the parent. Because the mutations in these strains appeared to have pleiotropic effects, they were not studied further.

In indirect inoculation assays, virulence was drastically affected by soil type. Tumorigenesis on plants grown in sand infested with the parent strain was the same as it was in direct inoculation assays, and there was only a slight reduction in mutant virulence. In soil, however, the virulence of even the parent strain was substantially reduced, to less than half the level of virulence in sand. This difference is presumably related to restricted motility of the bacteria in soil, which prevents the cells from reaching the root rapidly enough to incite tumors before wound healing occurs. Under such conditions, the loss of normal chemotactic capabilities appears to be a crucial limiting factor in crown gall pathogenesis on pea. The results indicate that chemotaxis may be important in some environments but is superfluous in others. This finding could explain why in one study de Weger et al. (12) found that chemotaxis in *P. fluorescens* was crucial to efficient root colonization, whereas in a separate study Howie et al. (20) found that nonmotile mutants of the *P. fluorescens* colonized roots as effectively as did the parent strain.

It is interesting that the wound-specific mutant C586 was avirulent in indirect soil assays. Apparently, attraction of this mutant to substances from wounds was not sufficient to overcome its loss of attraction to sloughed root cap cells. Ashby et al. (3) have reported a slight (three- to fivefold increase over background) but sensitive (10^{-8} M response threshold) Ti plasmid-mediated chemotactic attraction of *A. tumefaciens* C58C¹ to acetosyringone, a phenolic molecule released from wounds of some plants. On the basis of these data, the authors speculated that acetosyringone from wound sites in host plants may act as a signal in initial stages of recognition by *A. tumefaciens* (3). Not all strains are attracted to acetosyringone (18, 25), and Shaw et al. (28) recently suggested that even with strains like C58C¹, the response is likely to be important only on a "micrometre scale." Under the conditions of our assay, attraction to substances from nonwound exudates was apparently more important than chemicals released from wounds. We do not

yet know the chemical components of pea root exudates that attract *A. tumefaciens*.

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